Note

Molecular based method for the detection and quantification of larvae of the golden mussel *Limnoperna fortunei* using real-time PCR

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Abstract: A molecular based method for the quantitative detection of larvae of the golden mussel *Limnoperna fortunei* was developed. Partial nucleotide sequences of the mitochondrial cytochrome c oxidase subunit 1 (CO1) gene in several bivalve species including *L. fortunei* were analyzed. These nucleotide sequences and the CO1 gene for bivalve species found in habitats similar to *L. fortunei* (obtained from the EMBL/Genbank/DBJ databases) were aligned. Based on these results, *L. fortunei*-specific primers were designed for amplification. DNA extracted from several bivalve species including *L. fortunei* were subjected to PCR using *L. fortunei*-specific primers, and amplification of the DNA fragment was confirmed only in PCR assays which utilized template DNA from *L. fortunei*. No DNA fragments were amplified during the PCR of freshwater planktonic sample lacking golden mussel larvae which were collected from Lake Ohshio (Gunma, Japan). Real-time PCR was performed using fluorescent dye (SYBR Green) detection. Threshold cycles correlated well with the template DNA and number of larvae equivalents, and the R-square values of the standard curves were 0.99 and 0.98, respectively. These results suggest that this real-time PCR-based method can be utilized not only for the identification of *L. fortunei* in a particular habitat, but also for the quantification of larvae of this species in natural environments.

Key words: golden mussel, larvae, *Limnoperna fortunei*, quantitative detection, real-time PCR

The golden mussel *Limnoperna fortunei* (Dunker) is an invasive freshwater bivalve species that has been identified as contributing towards various ecological and economic problems (Darrigran et al. 1998, Ricciardi 1998, Penchaszadeh et al. 2000, Magara et al. 2001, Boltovskoy et al. 2006, Pie et al. 2006). In 2006, this species was designated as an Invasive Alien Species of Japan due to its adverse effects on ecosystems. During the life cycle of *L. fortunei*, the planktonic stage is an important phase that facilitates the distribution of this species. Given that the process of detecting larvae in planktonic samples is time-consuming, efficient quantitative detection methods are required. Pie et al. (2006) developed a sensitive method for detecting golden mussel larvae by employing a polymerase chain reaction (PCR)-based approach using a specific primer set: LIMNO.COIR1 and LIMNO.COIF1. Furthermore, Boeger et al. (2007) reported that this method allowed for the detection of 1–5 larvae. This PCR-based detection method, however, cannot be utilized for the accurate quantification of larval samples. Real-time PCR is recognized as an effective tool for the detection and quantification of planktonic organisms. In the present study, we used this technique for the

quantitative detection of golden mussel larvae.

Since the LIMNO.COIR1 and LIMNO.COIF1 primers (Pie et al. 2006) have not been optimized for real-time PCR assays, primers suitable for real-time PCR were newly designed. Adult *L. fortunei* were collected at Lake Ohshio (Gunma, Japan). Other bivalve species which were subjected to DNA extraction were collected in Lake Ohshio, Lake Hinuma (Ibaraki, Japan) and river mouth in Japan (see Table 1). The bivalves were fixed in 99.5% ethanol and stored at 4°C until used for DNA extraction. Muscle tissues were collected from each adult bivalve species, and DNA was extracted from each tissue sample using a DNeasy[®] blood and tissue kit (Qiagen, GmbH, Germany), according to the manufacturer's instructions. The region of the mitochondrial cytochrome c oxidase subunit 1 (CO1) gene was amplified by PCR using an Advantage[®] 2 PCR kit (Clontech Laboratories Inc., CA, USA) and the following primers (Folmer et al. 1994): 5'-GGTCAACAAATCATAAAGAT-ATTGG-3' (LCO1490) and 5'-TAAACTTCAGGGTGAC-CAAAAAATCA-3' (HCO2198). PCR was performed using the TaKaRa PCR Thermal Cycler Dice[®] Tp600 (TaKaRa Bio, Shiga, Japan), with initial denaturation at 95°C for 1 min, followed by 35 cycles of 95°C for 30 s, 54°C for 30 s, and 68°C for 30 s. Direct sequencing was performed on an ABI Prism[®] 310 genetic analyzer using a BigDye[®] Terminator Cycle Se-

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Table 1. Bivalve species used for DNA analysis in the present study.

Species	EMBL/Genbank/DDBJ accession number	Collection site	Collection date
<i>Limnoperna fortunei</i> (Dunker)	AB498011*	Lake Ohshio, Gunma, Japan	May 2008
<i>Xenostrobus securis</i> (Lamarck)	AB498012*	Lake Hamana, Shizuoka, Japan	April 2008
<i>Mytilus edulis</i> (Linnaeus)	NC006161	—	—
<i>Mytilus galloprovincialis</i> (Lamarck)	AB498013*	Daiba, Tokyo, Japan	May 2008
<i>Mytilus trossulus</i> (Gould)	AB498014*	Minamikayabe, Hokkaido, Japan	January 2009
<i>Perna viridis</i> (Linnaeus)	AB498015*	Daiba, Tokyo, Japan	September 2008
<i>Modiolus modiolus</i> (Linnaeus)	DQ264392	—	—
<i>Musculista senhousia</i> (Benson)	AB498016*	Daiba, Tokyo, Japan	May 2008
<i>Corbicula fluminea</i> (Müller)	AB498017*	Lake Ohshio, Gunma, Japan	October 2008
<i>Corbicula japonica</i> (Prime)	AB498018*	Lake Hinuma, Ibaraki, Japan	February 2009
<i>Crassostrea gigas</i> (Thunberg)	AF177226	—	—
<i>Crassostrea nippona</i> (Seki)	AF300616	—	—

* Nucleotide sequence data reported in this paper.

quencing Ready Reaction kit (Applied Biosystems, CA, USA) and the LCO1490 or HCO2198 primer. The nucleotide sequence data thus obtained were analyzed using ABI Prism[®] DNA Sequencing Analysis Software (Applied Biosystems). The nucleotide sequence data reported in the present study are available from the EMBL/Genbank/DDBJ databases under accession numbers AB498011–AB498018 (see Table 1). These nucleotide sequences and those of the CO1 gene for bivalve species found in habitats similar to those of *L. fortunei* (obtained from the EMBL/Genbank/DDBJ databases) were aligned using CLUSTAL X software (Jeanmougin et al. 1998). Species-specific sequences for *L. fortunei* were then selected, and following specific primers were designed for real-time PCR: 5'-TCTCTTCATTAGCTGGTGCCTC-3' (Limf-F) and 5'-CAAAACAGATGTAAGTCAAGAGAC-3' (Limf-R).

The specificity of the primer set was evaluated by performing PCR with DNA extracted from several bivalve species, or from planktonic sample containing or lacking golden mussel larvae as templates. Planktonic sample was collected from Lake Ohshio (Gunma, Japan) in August 20, 2008 using a plankton net (NXX13; diameter of 300 mm). The sample was fixed in 99.5% ethanol, and veligers of golden mussel were collected with microscopic observation. The remnant planktonic sample free from golden mussel larvae was subsequently used in experiments as planktonic sample lacking larvae. A planktonic sample (wet weight, 100 mg) containing 50 larvae, and planktonic sample (wet weight, 100 mg) lacking larvae were separately homogenized using a zirconia ball (YTZ[®] ball; Tosoh Corporation, Tokyo, Japan; 3 mm diameter). DNA was then extracted from homogenates using the Quick-Gene-Mini80 and Quick-Gene DNA tissue kit S (FUJIFILM Corporation, Tokyo, Japan), respectively, and subsequently employed as the templates in the PCR analysis. PCR was performed as described above with an annealing temperature of 60°C and PCR products were subjected to electrophoresis through a 2% agarose gel. Gels were then stained with SYBR Safe[™] DNA gel stain (Invitrogen Corporation, CA, USA). As a result, am-

plification of the DNA fragments was confirmed in PCR assays which utilized template DNA derived from *L. fortunei* and planktonic sample containing 50 larvae (Fig. 1). On the other hand, DNA fragments were not amplified when using template DNA derived from the other bivalve species or from the planktonic sample lacking larvae (Fig. 1).

Following this, the specific primer set was utilized for real-time PCR analysis of template DNA extracted from golden mussel adults (0.25, 0.5, 2.5, 5, 10, 25 and 50 ng DNA) and larvae (equivalent to 0.02, 0.04, 0.1, 0.2, 0.4, and 1 individuals). Template DNA was extracted from the larvae using the Quick Gene-Mini80 and Quick Gene DNA tissue kit S following homogenization with a zirconia ball. The real-time PCR was performed using fluorescent dye detection with the Thermal Cycler Dice[®] Real Time System (TP800; TaKaRa Bio). The amplification was carried out using SYBR[®] Premix Ex Taq[™] (TaKaRa Bio) under the following conditions: 95°C for 10 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The threshold cycle (Ct) values, the number of cycles corresponding to the threshold fluorescence, were measured, and standard curves were obtained from the Ct values. The Ct values correlated well with template DNA and number of larvae equivalents, and the R-square values of the standard curves were 0.99 and 0.98, respectively (Fig. 2). These results indicated that real-time PCR performed using Limf-F and Limf-R was effective for the quantification of *L. fortunei* DNA in natural planktonic samples.

In this paper, we have described a real-time PCR-based method that allows for the quantitative detection of golden mussel DNA with high sensitivity and precision. This method can be utilized not only for the identification of *L. fortunei* in a particular habitat, but also for the quantification of larvae of this species in natural environments.

Fig. 1. PCR amplification of the CO1 gene region in 8 bivalve species and planktonic samples containing or lacking golden mussel larvae, using golden mussel-specific primer sets. Lane M, 50–2500 bp DNA markers; lane 1, *Limnoperna fortunei*; lane 2, *Xenostrobus securis*; lane 3, *Mytilus galloprovincialis*; lane 4, *Mytilus trossulus*; lane 5, *Perna viridis*; lane 6, *Musculista senhousia*; lane 7, *Corbicula fluminea*; lane 8, *Corbicula japonica*; lane 9, planktonic sample containing 50 *L. fortunei* larvae; lane 10, planktonic sample lacking *L. fortunei* larvae.

Fig. 2. Real-time PCR standard curves of template DNA extracted from golden mussel adults (A) and larvae (B) using the golden mussel-specific primer set. The threshold cycle values were plotted against the log of the quantity of template DNA (ng) or the number of larvae in the reaction solution. Error bars indicate the standard error of the mean ($n=3$).

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